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DOI:
10.1101/gad.232009.113

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Genes Dev. 2014 28: 58-70
Access the most recent version at doi:10.1101/gad.232009.113

Targeting of MCL-1 kills MYC-driven mouse and human lymphomas even when they bear mutations in *p53*

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The transcriptional regulator c-MYC is abnormally overexpressed in many human cancers. Evasion from apoptosis is critical for cancer development, particularly c-MYC-driven cancers. We explored which anti-apoptotic BCL-2 family member (expressed under endogenous regulation) is essential to sustain c-MYC-driven lymphoma growth to reveal which should be targeted for cancer therapy. Remarkably, inducible Cre-mediated deletion of even a single *Mcl-1* allele substantially impaired the growth of c-MYC-driven mouse lymphomas. Mutations in *p53* could diminish but not obviate the dependency of c-MYC-driven mouse lymphomas on MCL-1. Importantly, targeting of MCL-1 killed c-MYC-driven human Burkitt lymphoma cells, even those bearing mutations in *p53*. Given that loss of one allele of *Mcl-1* is well tolerated in healthy tissues, our results suggest that therapeutic targeting of MCL-1 would be an attractive therapeutic strategy for MYC-driven cancers.

[*Keywords*: cancer; apoptosis; MCL-1; p53; BCL-2; MYC]

Supplemental material is available for this article.

Received October 1, 2013; revised version accepted November 19, 2013.

The c-MYC transcription factor regulates ~10,000 genes, including many that control cell growth and division (Dang 1999). Deregulated c-MYC expression is detected in up to 70% of human cancers, including lymphomas and leukemias (Boxer and Dang 2001; Sanchez-Beato et al. 2003). Much of what we know about c-MYC-driven tumorigenesis has emerged from studies using *Eμ-Myc* transgenic mice, in which the *c-Myc* transgene is subjugated to the immunoglobulin (*Ig*) heavy chain gene enhancer (*Eμ*), mimicking the consequences of the *c-MYC/IGH* or *c-MYC/IGL* chromosomal translocations that drive human Burkitt lymphoma (Adams et al. 1985). The *Eμ-Myc* mice exhibit a preleukemic expansion of pre-B cells that precedes the outgrowth of clonal sIg^- pre-B-cell or sIg^+ B-cell lymphomas arising with a median latency of ~110 d (Adams et al. 1985; Langdon et al. 1986).

Evasion from cell death is a hallmark of cancer, particularly those driven by c-MYC (Hanahan and Weinberg 2011; Kelly and Strasser 2011). Deregulated c-MYC expression enhances cell death under unfavorable growth conditions, such as limited availability of growth factors, through the intrinsic apoptotic pathway that is regulated by interactions of proteins belonging to three functionally distinct subgroups of the BCL-2 family (Youle and Strasser 2008). Following apoptotic stimuli, the proapoptotic BH3-only proteins (e.g., BIM and PUMA) become transcriptionally and/or post-transcriptionally up-regulated. For example, in response to DNA damage or oncogenic stress, the tumor suppressor p53 directly transcriptionally activates PUMA and NOXA to cause cell death (Jeffers et al. 2003; Villunger et al. 2003). The BH3-only proteins directly or indirectly activate BAX/BAK, the executioners of apoptosis, which permeabilize the outer

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Article is online at http://www.genesdev.org/cgi/doi/10.1101/gad.232009.113.

mitochondrial membrane and thereby unleash the downstream caspase cascade for cellular demolition (Merino et al. 2009; Llambi et al. 2011).

Apoptotic blocks, such as overexpression of prosurvival BCL-2-like proteins (Strasser et al. 1990; Swanson et al. 2004) or loss of proapoptotic BIM or PUMA (Egle et al. 2004; Hemann et al. 2004; Michalak et al. 2009), accelerate c-MYC-driven lymphomagenesis. Interestingly, mutations that deregulate the intrinsic apoptotic pathway, including amplifications of the genomic regions encoding *MCL-1* and *BCL-X* (Beroukhim et al. 2010), are detected in human tumors and are often associated with poor chemotherapy responses (Khaw et al. 2011). Burkitt lymphoma is associated with frequent loss of BIM and/or PUMA expression, in part due to gene hypermethylation (Mestre-Escorihuela et al. 2007; Garrison et al. 2008; Richter-Larrea et al. 2010; Giulino-Roth et al. 2012). Moreover, many Burkitt lymphomas contain defects in the p53 tumor suppressor pathway, particularly mutations in *p53* itself (Farrell et al. 1991; Bhatia et al. 1992) but also overexpression of the ubiquitin ligase HDM2 (called MDM2 in mice) or loss of the p14/ARF locus (Lindstrom et al. 2001).

Although overexpression of prosurvival BCL-2 family members accelerates c-MYC-induced lymphomagenesis, it is unclear whether the sustained survival and growth of c-MYC-driven lymphomas depends on the expression of these proteins under endogenous regulation. This remains an important question because knowledge of which prosurvival protein is essential for the sustained growth of a particular cancer will pinpoint the family member that should be targeted by the emerging BH3 mimetic drugs (Lessene et al. 2008). Using innovative gene targeting and drug-mimicking tools, we show for the first time that MCL-1 targeting kills c-MYC-driven mouse and human lymphoma cells even when p53 is mutated. This is remarkable given that we found that p53 does affect the dependency of these lymphoma cells on MCL-1 and suggest that therapeutic targeting of MCL-1 may be a promising strategy for cancer therapy.

Results

Experimental strategy to determine which anti-apoptotic BCL-2 family member is essential for the sustained survival and expansion of MYC-driven lymphomas

To examine the roles of BCL-X_L and MCL-1 expression under endogenous control in the sustained growth of *Eμ-Myc* lymphomas, we generated mice in which we could genetically delete *Mcl-1* or *Bcl-x* at will exclusively within c-MYC-driven lymphoma cells. Specifically, we produced *Eμ-Myc* transgenic mice that express a tamoxifen-regulated Cre recombinase-estrogen receptor fusion protein from the ubiquitously expressed Rosa26 locus (Rosa26-CreERT2 mice) (Seibler et al. 2003) and bear *loxP* targeted (*floxed*, denoted *fl*) alleles of the prosurvival gene *Bcl-x* (Wagner et al. 2000) or *Mcl-1* (Vikstrom et al. 2010) (Supplemental Fig. 1). As expected, the progeny with all three genetic alterations developed lymphomas with the same latency as standard *Eμ-Myc* mice (Supplemental Fig. 2A) because they expressed normal levels of BCL-X_L or MCL-1. The lymphoma cells were transplanted into C57BL/6-Ly5.1$^+$ recipients, which were treated with tamoxifen to activate the Cre-ERT2 recombinase, leading to deletion of *Bcl-x^{fl}* or *Mcl-1^{fl}* alleles exclusively within the malignant cells (Supplemental Fig. 1). Selected primary lymphomas were infected with an Ub-GFP-Luciferase lentivirus to facilitate monitoring lymphoma progression/regression following *Bcl-x* or *Mcl-1* deletion by imaging for bioluminescence in C57BL/6-albino recipient mice (Supplemental Fig. 1). In mice transplanted with *Eμ-Myc;CreERT2* lymphoma cells lacking any floxed allele (for simplicity, hereafter termed "control"), tumor expansion did not differ significantly between untreated and tamoxifen-treated recipients ($P = 0.06$) (Supplemental Fig. 2B). This shows that CreERT2 activation per se does not impair the growth of *Eμ-Myc* lymphomas.

Homozygous loss of Bcl-x only slightly impairs the sustained growth of Eμ-Myc lymphomas

Since endogenous BCL-X_L is necessary for *Eμ-Myc*-induced lymphoma development (Kelly et al. 2011), we predicted that it might also be essential for the sustained expansion of such malignant lymphomas. To examine this, primary lymphomas from nine *Eμ-Myc;CreERT2;Bcl-x$^{fl/fl}$* and seven control mice were transplanted into C57BL/6-Ly5.1$^+$ recipient mice and cohorts treated with tamoxifen. Homozygous *Bcl-x* loss resulted in only a modest delay in tumor expansion and slightly prolonged survival of the mice (median survival 25 d for *Eμ-Myc;CreERT2;Bcl-x$^{fl/fl}$* vs. 19 d for the controls; [*] $P = 0.0367$) (Fig. 1A). Following homozygous *Bcl-x* deletion, only 4% of the recipient mice (three from 73) showed complete lymphoma regression, which is comparable with the 3% of the tamoxifen-treated mice (one from 32) bearing control tumors.

This result was confirmed by in vivo lymphoma bioluminescence imaging (Fig. 1B). As early as 7 d after tumor transplant, the recipients displayed a significant lymphoma burden ($\geq 1 \times 10^7$ photon flux per second). These lymphomas continued to grow following tamoxifen treatment to delete both *Bcl-x* alleles and overwhelmed the recipients only shortly after the untreated mice had succumbed to the same lymphomas that retained *Bcl-x*. Efficient deletion of the *Bcl-x* alleles and loss of the BCL-X_L protein were confirmed by quantitative RT–PCR (qRT–PCR) and Western blot analyses in paired tamoxifen-treated and untreated lymphomas taken from mice transplanted with *Eμ-Myc;CreERT2;Bcl-x$^{fl/fl}$* lymphomas (Supplemental Fig. 3). These data reveal that malignant *Eμ-Myc* lymphomas can continue to grow without endogenous BCL-X_L expression.

Loss of Mcl-1 substantially impairs the sustained growth of Eμ-Myc lymphomas

In parallel experiments, we investigated whether MCL-1 was essential for the sustained growth of *Eμ-Myc* lymphomas. Cohorts of C57BL/6-Ly5.1$^+$ mice were transplanted with independent primary lymphomas from six

Eμ-Myc;CreERT2;Mcl-1$^{fl/fl}$ and seven control mice, and their survival was compared following tamoxifen treatment (Fig. 2). Strikingly, in 30% of the recipients, homozygous deletion of *Mcl-1* provoked complete lymphoma regression and extended survival (>180 d post-transplant) (Fig. 2A). The dramatic tumor regression was confirmed by bioluminescence imaging of lymphomas transplanted into C57BL/6-albino recipient mice (Fig. 2B). Importantly, even the tamoxifen-treated recipients that relapsed exhibited substantially extended survival compared with those bearing control lymphomas (median survival 35 d for mice bearing *Eμ-Myc;CreERT2;Mcl-1*$^{fl/fl}$ lymphomas vs. 19 d for those bearing control lymphomas; [****] $P < 0.0001$).

Remarkably, even heterozygous *Mcl-1*^{fl} loss substantially impaired sustained lymphoma growth: 20% of recipient mice bearing *Eμ-Myc;CreERT2;Mcl-1*$^{fl/+}$ lymphomas showed prolonged survival after tamoxifen treatment (median survival was 23 d for relapsing lymphomas vs. 19 d for control mice; [****] $P < 0.0001$) (Fig. 2A). These results demonstrate that the level of MCL-1 is highly critical for the sustained survival and expansion of MYC-driven lymphomas. This conclusion was strengthened by further analysis of the relapsing tamoxifen-treated lymphomas. The conditional allele of *Mcl-1* carries a human (hu) *CD4* reporter gene, which is expressed following *Mcl-1*^{fl} recombination, thus permitting single-cell analysis of the recombination efficiency (Vikstrom et al. 2010; Glaser et al. 2012). Strikingly, ~60% of *Eμ-Myc;CreERT2;Mcl-1*$^{fl/+}$ and 40% of *Eμ-Myc;CreERT2;Mcl-1*$^{fl/fl}$ lymphomas that relapsed following tamoxifen treatment had escaped deletion of their conditional *Mcl-1* alleles mostly because they had undergone selection for loss of CreERT2 expression (Fig. 3A,B). Western blot analysis of these tamoxifen-treated tumors confirmed that MCL-1 protein expression was not reduced (Fig. 3B). In addition, the minority of *Eμ-Myc;CreERT2;Mcl-1*$^{fl/fl}$ lymphomas that did relapse as huCD4-positive following tamoxifen treatment had only recombined one allele of *Mcl-1* and continued to express MCL-1 protein (Fig. 3C,D). By inference, the survival curves described earlier grossly underestimate the role that MCL-1 plays in the sustained growth of *Eμ-Myc* lymphomas. Indeed when animals that succumbed to relapsed lymphomas that had selected

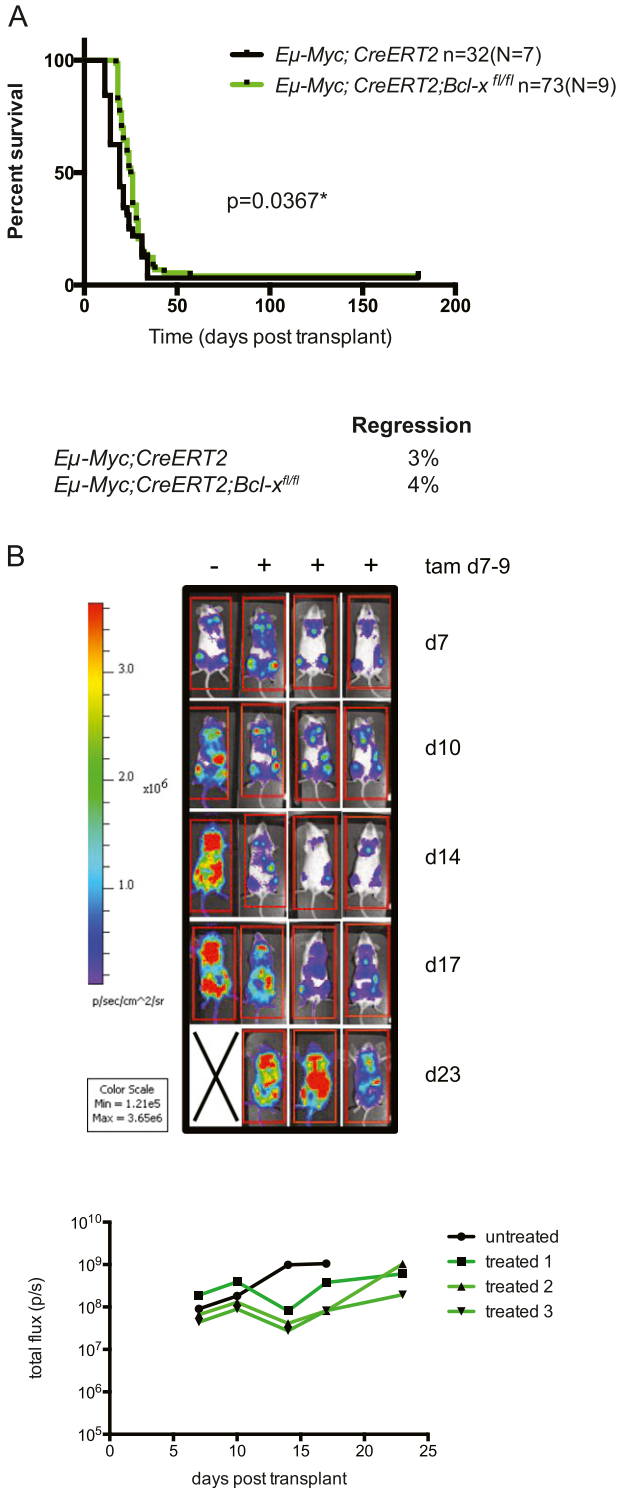
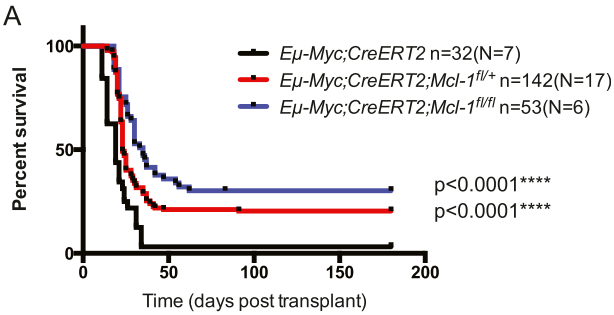
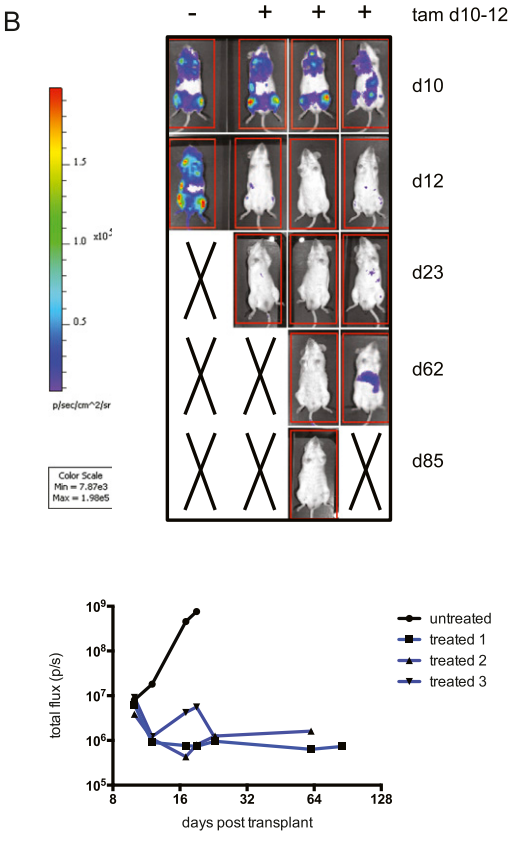


Figure 1. Homozygous loss of *Bcl-x* has only a minor impact on the growth of *Eμ-Myc* lymphomas. (*A*) Survival curves of C57BL/6-Ly5.1$^+$ recipient mice transplanted with *Eμ-Myc;CreERT2;Bcl-x*$^{fl/fl}$ (green line) or control (*Eμ-Myc;CreERT2*; black line) lymphoma cells and treated with tamoxifen to inactivate *Bcl-x* where applicable. (n) Total number of recipient mice analyzed; (N) number of independent lymphomas tested. Homozygous deletion of *Bcl-x* resulted in a small but significant delay in tumor growth. (*) $P = 0.0367$. For the mice transplanted with the control *Eμ-Myc;CreERT2* lymphomas, 3% regressed, and the overall median survival was 19 d. For the mice transplanted with the *Eμ-Myc;CreERT2;Bcl-x*$^{fl/fl}$ lymphomas, 4% regressed, and the overall median survival was 25 d. (*B*) Bioluminescence imaging of the tumor burden in C57BL/6-albino recipient mice injected with primary *Eμ-Myc;CreERT2;Bcl-x*$^{fl/fl}$ lymphoma cells that had been transduced with a lentiviral vector coexpressing GFP and luciferase. At 7 d post-transplant, a cohort of these mice was treated with tamoxifen. Mice were subsequently imaged for bioluminescence to monitor lymphoma burden every 3–4 d by measuring the total photon flux per second emitted from a region of interest (ROI) drawn around the whole mouse. See also Supplemental Figure 3.

against loss of *Mcl-1*^{fl} alleles were removed from the analysis (and those lymphomas that had mutated *p53* alleles were also removed from the survival curves) (Fig. 5A; see below), nearly 100% of animals survived lymphoma-free long-term (>180 d post-transplant) (Fig. 3E). These findings reveal that even heterozygous *Mcl-1* loss is highly detrimental for the sustained in vivo growth of malignant *Eμ-Myc* lymphomas, indicating that relatively weak targeting of MCL-1 might have therapeutic benefits in MYC-driven cancers.



	Regression
Eμ-Myc;CreERT2	3%
Eμ-Myc;CreERT2;Mcl-1$^{fl/+}$	20%
Eμ-Myc;CreERT2;Mcl-1$^{fl/fl}$	30%

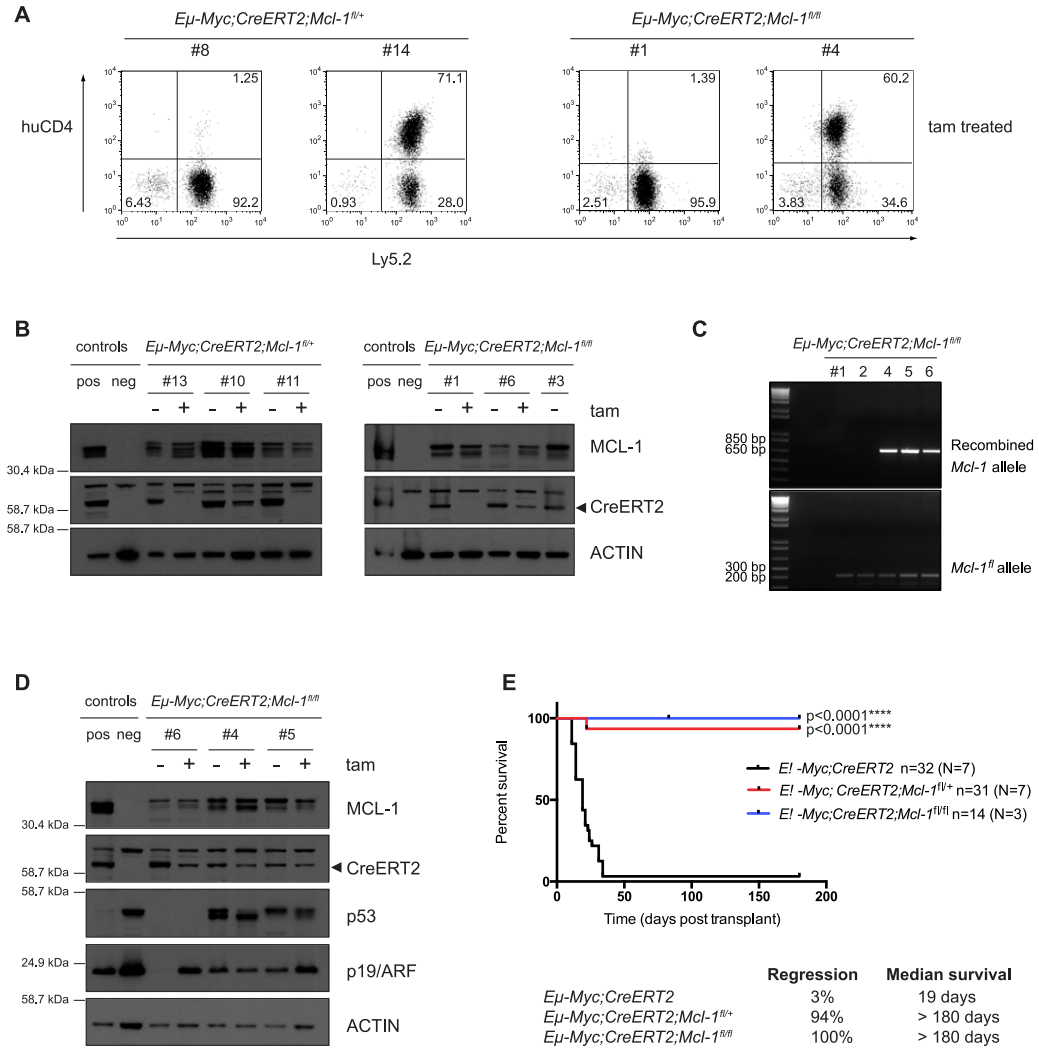


The sustained growth of human Burkitt lymphoma cells depends on the expression of MCL-1

We sought to translate these findings into the human disease setting. Western blot analysis of Burkitt lymphoma cell lines revealed that all expressed MCL-1 with heterogeneous expression of BCL-X_L and, as previously reported (Henderson et al. 1991) little to no BCL-2 (Fig. 4A). As a control for the BCL-2 antibody, we showed that the X50-7 and Awia-tr lymphoblastoid cell lines expressed high levels of this prosurvival protein (Fig. 4A), as reported (Henderson et al. 1991).

To examine the impact of targeting BCL-2 prosurvival proteins on Burkitt lymphoma growth, we developed a novel assay based on doxycycline (dox)-inducible expression (from a lentiviral vector) of genetically engineered variants of the BH3-only protein BIM_S with select binding specificities for distinct BCL-2 prosurvival proteins (Fig. 4B; Chen et al. 2005; Lee et al. 2008). These polypeptides have a mechanism of action similar to that of BH3 mimetic drugs; i.e., they antagonize BCL-2 prosurvival protein function by engaging a hydrophobic ligand-binding groove on their surface (Lee et al. 2007, 2008; Souers et al. 2013). The wild-type BIM_S, which binds with high affinity to all BCL-2 prosurvival proteins, served as a positive control for the integrity of the intrinsic apoptotic pathway. Mutant BIM_S4E, which does not bind to any BCL-2 prosurvival family member, thus served as a negative control. L62A/F69A mutant BIM_S2A preferentially binds MCL-1, and BIM_SBAD binds to BCL-2, BCL-X_L, and BCL-W. Western blotting verified inducible expression of all BIM_S variants in Rael-BL (Fig. 4C) and other Burkitt lymphoma cell lines (Supplemental Fig. 4A). All Burkitt lymphoma cell lines tested were highly sensitive to the expression of wild-type BIM_S, demonstrating that they had an intact intrinsic apoptotic pathway. Conversely, as expected, these cells were not affected by BIM_S4E (representative FACS plots are shown in Supplemental Fig. 4B).

Figure 2. Loss of *Mcl-1*, even loss of a single allele, greatly impairs the sustained growth of *Eμ-Myc* lymphomas within the whole animal. (*A*) Survival curves of C57BL/6-Ly5.1$^+$ recipient mice transplanted with *Eμ-Myc;CreERT2;Mcl-1*$^{fl/+}$ (red line), *Eμ-Myc;CreERT2;Mcl-1*$^{fl/fl}$ (blue line), or control (*Eμ-Myc;CreERT2*; black line) lymphoma cells and treated with tamoxifen to inactivate *Mcl-1* where applicable. (n) Total number of recipient mice analyzed; (N) number of independent lymphomas tested. Heterozygous and homozygous deletion of *Mcl-1* significantly delayed lymphoma growth. (****) $P < 0.0001$. For the mice transplanted with the control *Eμ-Myc;CreERT2* lymphomas, 3% regressed, and the overall median survival was 19 d. For the mice transplanted with the *Eμ-Myc;CreERT2;Mcl-1*$^{fl/+}$ lymphomas, 20% regressed, and the overall median survival was 23 d. For the mice transplanted with the *Eμ-Myc;CreERT2;Mcl-1*$^{fl/fl}$ lymphomas, 30% regressed, and the overall median survival was 35 d. (*B*) Bioluminescence imaging of the tumor burden in C57BL/6-albino recipient mice injected with primary *Eμ-Myc;CreERT2;Mcl-1*$^{fl/fl}$ lymphoma cells. At 10 d post-transplant, a cohort of these mice was treated with tamoxifen. These mice were subsequently imaged for bioluminescence.



	Regression	Median survival
Eμ-Myc;CreERT2	3%	19 days
Eμ-Myc;CreERT2;Mcl-1$^{fl/+}$	94%	> 180 days
Eμ-Myc;CreERT2;Mcl-1$^{fl/fl}$	100%	> 180 days

Figure 3. Most *Eμ-Myc* lymphomas that relapse following tamoxifen treatment have escaped *Mcl-1*^{fl} allele recombination. (*A*) FACS analysis to detect expression of huCD4 (reporter for *Mcl-1*^{fl} recombination) on the surface of two representative *Eμ-Myc;CreERT2;Mcl-1*$^{fl/+}$ and *Eμ-Myc;CreERT2;Mcl-1*$^{fl/fl}$ lymphomas that had relapsed in recipient mice after tamoxifen treatment (transplanted lymphoma cells stained positive for Ly5.2). Approximately 60% of the relapsed *Eμ-Myc;CreERT2;Mcl-1*$^{fl/+}$ and 40% of the relapsed *Eμ-Myc;*CreERT2;*Mcl-1*$^{fl/f}$ lymphoma cells had escaped *Mcl-1*^{fl} allele recombination and, as such, were huCD4-negative (like the sample at the *left* of each pair), whereas ~40% of the relapsed *Eμ-Myc;*CreERT2;*Mcl-1*$^{fl/+}$ and 60% of the relapsed *Eμ-Myc;CreERT2;Mcl-1*$^{fl/fl}$ lymphomas had efficiently recombined at least one *Mcl-1*^{fl} allele, as reflected by staining positive for huCD4 (like the sample at the *right* of each pair). (*B*) Immunoblotting to detect MCL-1 and CreERT2 protein expression (probing for Actin was used as a loading control) in extracts from the spleens, lymph nodes, or thymuses of sick mice that had been transplanted with *Eμ-Myc;CreERT2;Mcl-1*$^{fl/+}$ or *Eμ-Myc;*CreERT2;*Mcl-1*$^{fl/fl}$ lymphomas. Three independent, paired control, and tamoxifen-treated tumors of each genotype were analyzed (note that one of the *Eμ-Myc;CreERT2;Mcl-1*$^{fl/fl}$ tumors [#3] examined never relapsed after tamoxifen treatment). *Mcl*-1 knockout (KO) mouse embryonic fibroblasts (MEFs) were used as a control to confirm the specificity of the MCL-1 antibody (neg control). (*C*) DNA PCR analysis of recombined *Mcl-1*^{fl} and unrecombined (intact) floxed alleles in *Eμ-Myc;CreERT2;Mcl-1*$^{fl/fl}$ lymphomas that relapsed following tamoxifen treatment. (*D*) Immunoblotting to detect MCL-1, CreERT2, p53, p19/ARF and Actin (loading control) protein expression in extracts from the spleens, lymph nodes, or thymuses of sick mice transplanted with *Eμ-Myc;CreERT2;Mcl-1*$^{fl/fl}$ lymphomas that relapsed as huCD4-positive following tamoxifen treatment. Three independent, paired control, and tamoxifen-treated tumors were analyzed. *Mcl-1* knockout (KO) MEFs were used as a control (neg control) to confirm the specificity of the MCL-1 antibody. (*E*) Survival curves of C57BL/6-Ly5.1^{+} recipient mice transplanted with *Eμ-Myc;CreERT2;Mcl-1*$^{fl/+}$ (red line) or *Eμ-Myc;CreERT2;Mcl-1*$^{fl/fl}$ (blue line) lymphoma cells that had wild-type *p53* genes following tamoxifen treatment and excluding those lymphomas that escaped Cre-mediated deletion of one or both *Mcl-1*^{fl} alleles. C57BL/6-Ly5.1^{+} recipient mice transplanted with control (*Eμ-Myc;CreERT2*; black line) lymphoma cells and treated with tamoxifen are shown for comparison. (n) Total number of recipient mice analyzed; (N) number of independent lymphomas tested. Efficient heterozygous and homozygous deletion of *Mcl-1* significantly delayed lymphoma growth. (****) $P < 0.0001$. For the mice transplanted with the control *Eμ-Myc;CreERT2* lymphomas, 3% regressed, and the overall median survival was 19 d. For the mice transplanted with the *Eμ-Myc;CreERT2;Mcl-1*$^{fl/+}$ lymphomas, 94% regressed, and the overall median survival was >180 d. For the mice transplanted with the *Eμ-Myc;CreERT2;Mcl-1*$^{fl/fl}$ lymphomas, 100% regressed, and the overall median survival was >180 d.

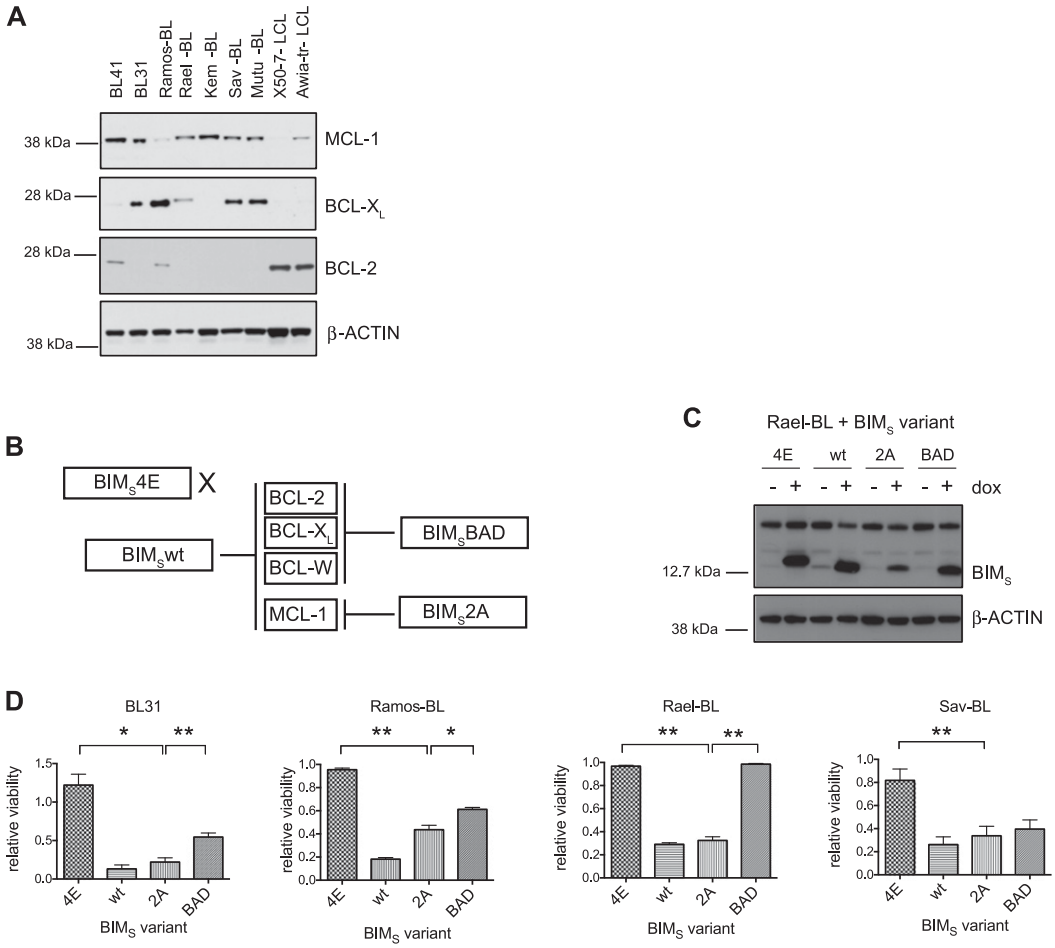


Figure 4. MCL-1 blockade kills human Burkitt lymphoma cells, but some also show minor dependency on BCL-X_L. (*A*) Immunoblotting to detect MCL-1, BCL-X_L, BCL-2, and β-Actin (loading control) in extracts from Burkitt lymphoma cell lines and, as controls for BCL-2 expression, the X50-7 and Awia lymphoblastoid cell lines. (*B*) Schematic showing the binding specificities of the BIM_S variants to the BCL-2 prosurvival proteins. (*C*) Immunoblotting to detect the inducible expression of the BIM_S variants before and after 24 h of dox treatment in the Rael-BL cell lines carrying the different lentiviral-inducible BIM_S expression constructs. Cells were maintained in medium supplemented with the pan-caspase inhibitor qVD-OPh to prevent protein degradation due to apoptosis. Note that the endogenous BIM_{EL}, BIM_L, and BIM_S proteins can also be detected but at a lower level. Probing for β-Actin served as a loading control. (*D*) Viability of BL cell lines stably infected with lentiviral constructs carrying vectors for dox-inducible expression of BIM_S variants was determined 72 h after the addition of dox to the medium by staining the cells with propidium iodide (PI) followed by FACS analysis for PI and GFP fluorescence (GFP is expressed from the lentivirus encoding the BIM_s variants). The PI-negative/GFP-positive viable cells were recorded. The percentage of viable/GFP-positive untreated cells was assigned an arbitrary value of 1, and the percentage of viable/GFP-positive dox-treated cells was expressed as a proportion of this. Each line was assayed in triplicate, and data are presented as the mean and standard error of the mean of three independent experiments. Statistical analysis using a paired two-tailed *t*-test showed that BIMs2A induced significantly more death in all of the Burkitt lymphoma cells examined compared with the negative control BIMs4E (BL31, [*] $P = 0.0179$; Ramos-BL, [**] $P = 0.0025$; Rael-BL, [**] $P = 0.0017$; Sav-BL, [**] $P = 0.0044$) and that BIMs2A induced significantly more death than BIMsBAD in BL31 ([**] $P = 0.0056$), Ramos-BL ([*] $P = 0.0421$), and Rael-BL ([**] $P = 0.0023$) cells but not in the Sav-BL cells ([ns] $P = 0.1348$). See also Supplemental Figure 4.

Notably, all Burkitt lymphoma cells were sensitive to BIM_S2A, indicating a dependency on MCL-1 (Fig. 4D; Supplemental Fig. 4D). Ramos-BL, BL31, and Sav-BL were also sensitive to BIM_SBAD (and, accordingly, also to treatment with ABT-737, which targets BCL-X_L, BCL-2, and BCL-W), albeit for Ramos-BL and BL31, significantly less than to BIM_S2A, indicating only a partial dependency on BCL-X_L (Fig. 4D; Supplemental Fig. 4C,D). Kinetic binding analyses using the Biacore confirmed that the more potent killing activity of BIM_S2A versus BIM_SBAD on most cell lines reflected the dependence of these cells on MCL-1 rather than a greater capacity of BIM_S2A to engage MCL-1 compared with the ability of BIM_SBAD to bind BCL-X_L. Indeed these Biacore binding assays showed that the affinity of the BAD-BH3 is actually ~20-fold higher for BCL-X_L (similar to that of wild-type BIM_S BH3) than the affinity of BIM_S2A for MCL-1 due to a significantly faster dissociation rate in the MCL-1 interaction

(Supplemental Fig. 4E). Collectively, these results show that the sustained survival and growth of Burkitt lymphoma cells is dependent on MCL-1 either alone or with a contribution by BCL-X_L.

Mutations in p53 reduce but do not ablate the dependency of c-MYC-driven mouse and human lymphomas on MCL-1

Although most transplanted *Eμ-Myc* mouse lymphomas (14 from 23 analyzed) could not continue to grow following heterozygous recombination of *Mcl-1*^{fl}, a minority of relapsing lymphomas (nine from 23 analyzed) did display heterozygous *Mcl-1*^{fl} allele deletion, as demonstrated by huCD4 immunostaining (Fig. 3A). We were interested to understand why these lymphoma cells were less dependent on MCL-1 for their growth, since such insights will be critical for predicting the therapeutic response of c-MYC-driven lymphomas and other cancers to MCL-1 antagonists. Interestingly, we noticed that most lymphomas capable of growth following transplantation and heterozygous *Mcl-1*^{fl} loss (seven of nine identified) displayed dramatically increased expression of the tumor suppressor p53 (Figs. 3D, 5A), a hallmark of *p53* gene mutations. DNA sequence analysis confirmed the presence of mutations affecting known "hot spot" residues (Freed-Pastor and Prives 2012) within the DNA-binding domain of p53 in the primary lymphomas capable of growth following transplantation and heterozygous deletion of *Mcl-1* (Figs. 3D, 5B). This suggests that the presence of existing mutations in the *p53* gene decreased the dependency of these tumor cells on MCL-1. An additional relapsed lymphoma of genotype *Eμ-Myc; CreERT2;Mcl-1*$^{fl/fl}$ (#6) showed evidence of p53 pathway mutation (increased p19/ARF protein expression) upon tamoxifen treatment (increasing the frequency to eight of nine lymphomas showing perturbation of the p53 pathway) (Fig. 3D). As a control, we sequenced the p53 gene in seven of the 14 lymphomas that expressed physiological (i.e., very low/barely detectable in the absence of genotoxic stress) levels of p53 (indicative of wild-type nonmutated *p53* genes) and were not capable of growing following heterozygous loss of *Mcl-1* and confirmed that their *p53* genes were wild type. This highlights a novel and highly specific interplay between malignant cell survival dependency on MCL-1 and the p53 tumor suppressor pathway. Importantly, a lymphoma of genotype *Eμ-Myc;CreERT2;Mcl-1*$^{fl/fl}$ (#3) carrying the *p53* mutation that introduced a stop codon almost always regressed following tamoxifen treatment. These results suggest that mutations in the p53 pathway and complete loss of wild-type p53 were not sufficient to allow sustained expansion of *Eμ-Myc* lymphomas with homozygous *Mcl-1* loss.

Therefore, and since mutations in *p53* are a common feature of many human cancers (Vousden and Lane 2007), we investigated whether p53 pathway defects could render Burkitt lymphoma cells resistant to MCL-1 targeting. Mutations of the *p53* gene, clustering around "hot spot" residues in the DNA-binding domain that are known to abrogate p53 function (Cho et al. 1994; Petitjean et al. 2007; Freed-Pastor and Prives 2012), were detected in Rael-BL, Sav-BL, and BL-31 and globally in ~60% of the 16 Burkitt lymphoma cell lines tested (note that many lines were in early passage) (Fig. 5C,D). Importantly, all Burkitt lymphoma cells that retained wild-type *p53* genes displayed other abnormalities in the p53 tumor suppressor pathway, including HDM2 overexpression (Fig. 5C) or p14ARF locus deletion (BL2) (Fig. 5D). Thus, functional inactivation of the p53 tumor suppressor pathway appears to constitute a critical step in the pathogenesis of c-MYC-driven Burkitt lymphoma but is insufficient to overcome the exquisite growth dependency of these malignant cells on MCL-1. Therefore, therapeutic targeting of MCL-1 would still remain an effective treatment regime for Burkitt lymphoma and possibly other c-MYC-driven human cancers.

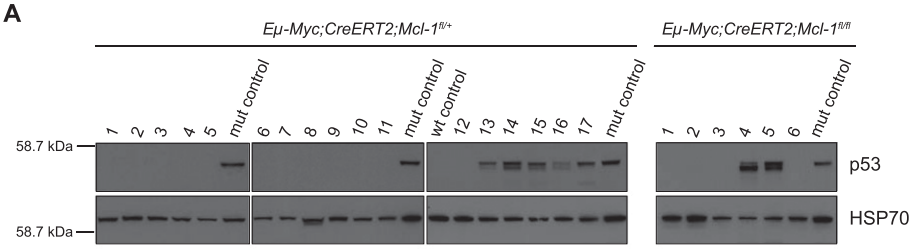
Discussion

Up to 70% of human cancers display deregulated c-MYC overexpression (Boxer and Dang 2001; Sanchez-Beato et al. 2003). Identifying the cellular factors critical for the growth of c-MYC-driven cancers therefore remains an important objective for designing novel treatment strategies.

We investigated this by using two novel, innovative approaches: conditional deletion of *Mcl-1* or *Bcl-x* genes at will in *Eμ-Myc* lymphomas within the whole mouse or inducible expression of polypeptides that inhibit specific BCL-2 prosurvival proteins (mimicking the action of drugs that target these proteins; e.g., ABT-737 or ABT-263/navitoclax) in human Burkitt lymphoma cells. We found that although BCL-X_L is essential for *Eμ-Myc*-induced lymphoma development (Kelly et al. 2011), the loss of BCL-X_L had only a minimal impact on sustained tumor growth. Consistent with this, treatment of *Eμ-Myc* lymphomas with the BH3 mimetic ABT-737, which targets BCL-2, BCL-X_L, and BCL-W (Oltersdorf et al. 2005; van Delft et al. 2006), does not cause tumor regression (Mason et al. 2008), suggesting that these three prosurvival proteins are dispensable for sustained *Eμ-Myc* lymphoma growth. In contrast, sustained growth of *Eμ-Myc* lymphomas was exquisitely dependent on MCL-1. Remarkably, even heterozygous deletion of *Mcl-1* was sufficient to result in complete regression of ~20% of tumors, allowing long-term survival of formerly lymphoma-burdened mice. Strikingly, analysis of the lymphomas that did relapse revealed that ~60% had escaped *Mcl-1* deletion (mostly due to loss of the Cre recombinase). Indeed, when the animals bearing lymphomas that escaped *Mcl-1*^{fl} allele deletion (and had not acquired mutations in p53) were removed from the analysis, nearly 100% survived lymphoma-free long-term (>180 d post-transplant) (Fig. 3E). Importantly, this translated into the human disease setting, where we found that MCL-1 blockade efficiently killed Burkitt lymphoma cells, with a lesser impact of BCL-X_L targeting observed in some lines.

An MCL-1 dependency was also detected in AML mouse models and human AML-derived cell lines (Xiang

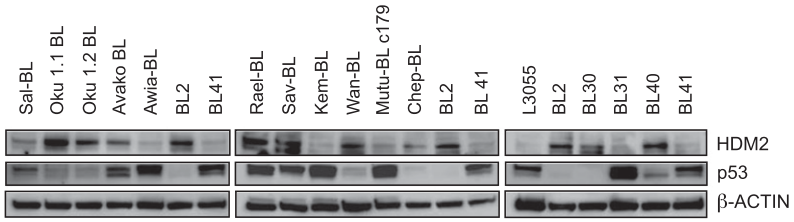
A



B

Mouse genotype	Mouse *p53* mutation	Homozygous/ heterozygous mutation	Frequency (No. of tumors)	Equivalent affected human *p53* aa
Eμ-Myc;CreERT2;Mcl-1$^{fl/+}$	V170M	hom	1	V173
	I192S	het	1	I195
	R246Q	hom	1	R249
	R270H	hom	1	R273
	280G insertion	hom	1	R283
Eμ-Myc;CreERT2;Mcl-1$^{fl/fl}$	R270H	hom	2	R273
	S224 (deletion of 2bps)	hom	1	S227

C



D

BL cell line	p53 gene mutation	HDM2 expression	p14^{ARF} locus
Sal-BL	WT	Mid	Intact
Oku-BL	WT	High	Intact
Wan-BL	WT	High	Intact
Chep-BL	WT	Mid	Intact
BL2	WT	High	Deleted
BL30	WT	High	Intact
BL40	WT	High	Intact
Rael-BL	R282W	High	Intact
Kem-BL		Low	Intact
L3055		Low	Intact
BL31	V172F	Low	Intact
Sav-BL	G244D	High	Intact
Mutu-BL cl 179	G245D	Low	Intact
Ava-BL	M246T	Mid	Intact
BL41	R248Q	Low	Intact
Awia-BL	Deletion of Q165, S166, Q167	Low	Intact

Figure 5. Mutations in *p53* reduce but do not ablate the dependency of c-MYC-driven mouse and human lymphomas on MCL-1. (*A*) Immunoblotting to detect stabilized p53 proteins, indicative of a mutant p53 protein, in extracts from 17 *Eμ-Myc;CreERT2;Mcl-1*$^{fl/+}$ and six *Eμ-Myc;CreERT2;Mcl-1*$^{fl/fl}$ lymphomas. All but two of the samples were extracted from transplanted and tamoxifen-treated lymphomas. The two exceptions were *Eμ-Myc;CreERT2;Mcl-1*$^{fl/+}$ #9, which was a primary lymphoma, and *Eμ-Myc;CreERT2;Mcl-1*$^{fl/fl}$ #3, which was a transplanted but not tamoxifen-treated lymphoma (this particular lymphoma has the deletion of two bases that introduces a premature stop codon in the *p53* gene). Probing for HSP70 served as a loading control. (*B*) Summary of the *p53* mutations detected in *Eμ-Myc;CreERT2;Mcl-1*$^{fl/+}$ and *Eμ-Myc;CreERT2;Mcl-1*$^{fl/fl}$ lymphomas as detected by DNA sequence analysis. The mutations are listed as wild-type (wt) amino acid (aa) affected, amino acid position, and mutant amino acid. The frequencies at which the mutations were detected in the lymphomas and the human equivalents of the mutations are listed. The sequence alignment for murine *p53* was performed using Ensembl transcript number ENSMUST00000108658 as the reference transcript. (*C*) Immunoblotting to detect the expression of HDM2, p53, and β-Actin (loading control) in human Burkitt lymphoma cell lines. (*D*) Summary of the *p53* pathway aberrations detected in the human Burkitt lymphoma cell lines. The *p53* mutations present in each cell line is listed as wild-type (wt) amino acid (aa) affected, amino acid position, and mutant amino acid, and the number of alleles affected is also detailed.

et al. 2010; Glaser et al. 2012). This finding and the observation that ~10% of diverse tumors contain somatically acquired amplifications of the genomic region harboring *MCL-1* (Beroukhim et al. 2010) highlight the importance of developing BH3 mimetic drugs that selectively target MCL-1 for treating human cancers. A concern is that MCL-1 is required for the survival of hematopoietic stem/progenitor cells (Opferman et al. 2005) and several other critical cell types, including cardiomyocytes (Opferman et al. 2003; Vikstrom et al. 2010; Thomas et al. 2013; Wang et al. 2013). Importantly, our discovery that MYC-driven lymphomas cannot tolerate even heterozygous loss of *Mcl-1* (which should mimic 50% drug-mediated inhibition of the protein), whereas its heterozygous loss is well tolerated in normal tissues (Opferman et al. 2003, 2005; Vikstrom et al. 2010), indicates that it should be possible to establish a therapeutic window for MCL-1 inhibitory drugs.

A novel and highly important finding from this study is the observation that mutations in "hot spot" residues in the DNA-binding domain of the *p53* gene can reduce but not abrogate the lymphoma cells' dependency on MCL-1. Consistent with a recent study that found that *p53* mutations are the fourth most commonly detected genetic change in a large panel of Burkitt lymphoma biopsies (Love et al. 2012), we found that ~60% of Burkitt lymphoma lines had acquired mutations in the DNA-binding domain of *p53* (Petitjean et al. 2007). Remarkably, all of the remaining Burkitt lymphoma lines examined displayed some other abnormalities in components of the p53 pathway, such as HDM2 overexpression or p14/ARF loss. Importantly, MCL-1 antagonism was able to kill Burkitt lymphomas with p53 pathway defects, suggesting that MCL-1 inhibitory drugs could be efficacious in the treatment of c-MYC-driven cancers bearing *p53* mutations.

The network of processes that p53 activates to suppress tumorigenesis are still emerging (Brady et al. 2011; Li et al. 2012; Valente et al. 2013). The observation that mutation and loss of *p53* constitute critical steps in c-MYC-driven lymphomagenesis (Vousden and Lane 2007; Michalak et al. 2009) and that they can facilitate the sustained growth of malignant *Eμ-Myc* lymphomas with reduced MCL-1 expression highlights the connection between the p53 tumor suppressor pathway and the dependency of malignant cells on prosurvival MCL-1.

Our results are consistent with a model (Fig. 6) in which c-MYC-driven lymphomas are highly dependent on MCL-1 for survival. A reduction of MCL-1 expression through either genetic means or, in the future, therapeutics could induce cell death by disturbing the balance between the proapoptotic p53 targets PUMA/NOXA and prosurvival MCL-1. However, when designing therapeutic strategies for such lymphomas, we need to be mindful that mutations in *p53* would prevent oncogenic stress-induced up-regulation of PUMA and NOXA and may thereby render lymphoma cells less dependent on MCL-1. Therefore, for such (*p53* mutated) c-MYC-driven lymphomas, it may be beneficial to activate the intrinsic apoptotic pathway more potently than is needed for tumors that carry wild-type *p53*.

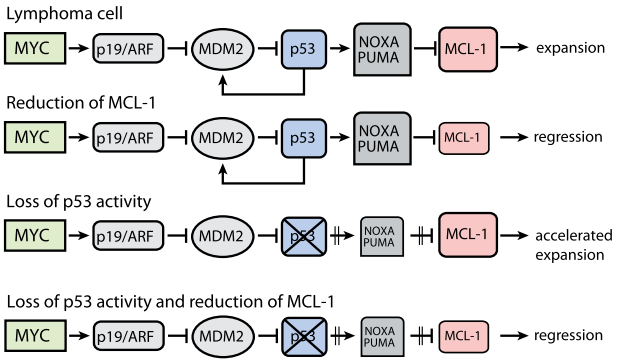


Figure 6. A proposed model in which c-MYC-driven lymphoma cells that are highly dependent on MCL-1 for their sustained expansion can no longer survive once MCL-1 expression is reduced and the balance between this prosurvival protein and the *p53* proapoptotic targets PUMA/NOXA is disturbed. The c-MYC-driven lymphoma cells that have acquired *p53* mutations display an accelerated lymphoma progression. These lymphomas express less PUMA/NOXA, and, consequently, less MCL-1 expression is required to maintain the lymphoma progression. Despite this, we showed that targeting of MCL-1 in lymphomas with *p53* mutations was sufficient to result in tumor regression. Note that the mouse protein nomenclature is used.

In conclusion, our findings suggest that MCL-1 targeting may be a viable strategy for cancer therapy, particularly in tumors where deregulated c-MYC expression instills an exquisite dependence (much more so than in nontransformed cells) on this prosurvival protein.

Materials and methods

Mice

Experiments with mice were conducted according to the guidelines of The Walter and Eliza Hall Institute Animal Ethics Committee. *Eμ-Myc* transgenic (Adams et al. 1985), *Bcl-x*$^{fl/fl}$ (Wagner et al. 2000), *Mcl-1*$^{fl/fl}$ (Vikstrom et al. 2010), *Rosa26-CreERT2* (Seibler et al. 2003), and *p53*$^{-/-}$ (Jacks et al. 1994) gene targeted mice have all been described previously. All mouse strains were on a C57BL/6-Ly5.2^{+} genetic background generated on either this background (*Mcl-1*$^{fl/fl}$ and *Rosa26-CreERT2*) using C57BL/6-derived embryonic stem cells, a mixed C57BL/6x129SV background (*Bcl-x*$^{fl/fl}$) using 129SV-derived embryonic stem cells, or a mixed C57BL/6xSJL background (*Eμ-Myc*) using microinjection of C57BL/6xSJL-derived oocytes and then backcrossed for 10 to >20 generations with C57BL/6 mice.

Single-cell suspensions of 3×10^6 *Eμ-Myc* lymphoma cells in PBS were injected into C57BL/6-Ly5.1^{+} or C57BL/6J-Tyr$^{c\text{-}2J}$ (referred to as C57BL/6-albino; The Jackson Laboratory) recipient mice by intravenous (i.v.) tail vein injection. Mice were administered by oral gavage with 200 mg/kg tamoxifen (Sigma-Aldrich) in peanut oil/10% ethanol per day (Anastassiadis et al. 2010) for two consecutive days on either days 5 and 6 or days 10 and 11 post-injection of the tumor cells. Transplants in which the matched control untreated mice did not become sick by 28 d post-tumor cell injection were excluded from the analysis.

Statistical analysis

Graphpad Prism software was used for generating Kaplan-Meier plots and performing statistical analysis (using a log-rank test) to compare the survival of mice injected with lymphoma cells of different genotypes. Graphpad Prism was also used to carry out paired two-tailed *t*-tests on the death induced in the Burkitt lymphoma cells following dox-inducible expression of the BIMs variants. Specifically, the significance of the extent of cell death induced by BIMs2A (to target MCL-1) expression compared with either inducible expression of BIMs4E (negative control) or BIMsBAD (to target BCL-2, BCL-X_L, and BCL-W) was calculated for each of the Burkitt lymphoma cell lines.

Cell culture

Eμ-Myc mouse lymphoma cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco), 50 μM β-mercaptoethanol (Sigma-Aldrich), 100 μM asparagine (Sigma-Aldrich), 100 U/mL penicillin, and 100 mg/mL streptomycin (Gibco) at 37°C and 10% CO_2. OP9 cells were cultured in αMEM (Gibco) supplemented with 20% heat-inactivated FBS, 1 mM glutamine (Gibco), 10 mM Hepes (Gibco), 1 mM sodium pyruvate (Gibco), 50 μM β-mercaptoethanol, 100 U/mL penicillin, and 100 μg/mL streptomycin. Human cell lines were cultured in a humidified incubator at 37°C and 5% CO_2. Virus-producing 293T cells were maintained in DMEM supplemented with 10% FBS. Approximately 6 h prior to transfection, 293T cells were cultured in DME glutamax (Gibco) supplemented with 10% FBS and 25 mM Hepes. Rael-BL, Ramos-BL, Sav-BL, and BL-31 human Burkitt lymphoma-derived cell lines were cultured in RPMI 1640 supplemented with 10% FBS, 1 mM glutamine (Gibco), 1 mM sodium pyruvate (Gibco), 50 μM α-thioglycerol (Sigma-Aldrich), and 20 nM bathocuproine disulfonic acid (BCS) (Sigma-Aldrich). X50-7 and Awia lymphoblastoid cell lines were cultured in RPMI 1640 supplemented with 10% FBS and 1 mM glutamine.

Lentiviral plasmids and virus production

The dox-responsive lentiviral vector pFTRE3G_pGK3G_GFP was generated by digesting PacI/AscI and cloning the TRE3G_pGK3G_GFP cassette. The TRE3G_pGK3G GFP cassette was amplified by PCR as pTRE3G_pGK3G_GFP (Yamamoto et al. 2012). The PCR products of the BIM_S variants (Lee et al. 2008) were cloned into pFTRE3G_pGK3G_GFP. The lentiviral imaging construct was generated by inserting Luciferase2 linked via a T2A peptide to eGFP downstream from the human ubiquitin promoter of the FUGW vector (Lois et al. 2002). Lentivirus-containing supernatants were produced by transiently transfecting 293T cells with the expression constructs of interest alongside the packaging plasmids, VSV-G, MDL, and RSV-Rev using the standard $CaPO_4$ method (Herold et al. 2008). Supernatants containing infectious virus particles were harvested 2–3 d post-transfection.

Lentiviral transduction of Eμ-Myc lymphoma cells and Burkitt lymphoma cell lines

For infection, aliquots of 0.5×10^6 to 1×10^6 primary *Eμ-Myc* lymphoma cells or 1×10^5 Burkitt lymphoma cells were suspended in 4 mL of virus-containing supernatant containing 10 ng/μL polybrene, incubated for 30 min at 37°C and 5% or 10% CO_2, and then centrifuged at 2200 rpm for 2.5 h at 32°C. The supernatant was subsequently discarded, and the cells were resuspended in fresh medium for culture.

Inducible expression of BIM_S variants in human Burkitt lymphoma cells

To induce expression of the BIM_S variants, cells were cultured in medium supplemented with 1 μg/mL dox. The viability of the GFP-positive cells was determined by propidium iodide (PI) staining and FACS analysis in an LSR1 machine. The PI-negative/GFP-positive cells were considered as live cells. The cell death following inducible expression of the BIMs variant was expressed relative to the untreated Burkitt lymphoma cells, which were assigned a value of 1. For the analysis of the BIM_S variant expression by immunoblotting, the pan-caspase inhibitor qVD-OPh (25 μM; MP Biomedicals) was added to the dox-treated cells for 24 h to inhibit cell killing.

Direct binding assays to determine the binding parameters of BH3 ligands for BCL-X_L and MCL-1

Direct binding assays were performed at room temperature using a Biacore S51 biosensor exactly as described previously (Lee et al. 2007). The peptides used were BIMs BH3, DMRPEIWIAQELRR IGDEFNAYYARR; BIM_S 2A BH3, DMRPEIWIAQEARRIGDEA NAYYARR; and BAD_S BH3, NLWAAQRYGRELRRMSDEFVD SFKKG.

Immunofluorescent staining, flow cytometric analysis, and cell sorting

To immunophenotype *Eμ-Myc* lymphomas, single-cell suspensions were stained as described (Strasser et al. 1991) with surface marker-specific antibodies and analyzed using an LSR1 machine (Becton Dickinson). The following fluorochrome-conjugated antibodies were used: CD19 (clone ID3), B220 (RA3-6B2), IgM (clone 5.1), IgD (clone 11-26C), CD4 (clone H129), CD8 (clone YTS.169), Thy1 (clone T3.24.1), Mac1 (M1/70), and GR1 (clone RB6-8C5). To discriminate host-derived ($Ly5.1^+$) from donor-derived ($Ly5.2^+$) cells, monoclonal antibodies to Ly5.1 (clone A201.1) and Ly5.2 (clone 5.450.15.2 or AL14A7) were used. To verify recombination of the *loxP*-flanked allele of *Mcl-1*, cells were stained with antibodies to human CD4 (clone RPA-T4), since $Mcl\text{-}1^{fl}$ recombination subjugates a human CD4 reporter cassette to the *Mcl-1* promoter/enhancer (Vikstrom et al. 2010). Antibodies were produced in our laboratory and conjugated to FITC, R-phycoerythrin (R-PE), or allophycocyanin (APC) according to the manufacturers' instructions. Where required, Burkitt lymphoma cells were sorted for GFP expression using an AriaW cell sorter (Becton Dickinson).

Immunoblotting

Total protein extracts were prepared from cells (primary *Eμ-Myc* lymphomas and Burkitt lymphoma cell lines) by lysis in onyx buffer, NP40 buffer, or urea buffer containing protease inhibitors (Complete protease inhibitor cocktail, Roche). Protein content was quantified using the Bio-Rad Bradford assay. Total protein extracts (20 μg) were separated on the basis of molecular weight by SDS-PAGE and Western-blotted onto nitrocellulose membranes. The membranes were blocked in 5% skim milk in PBS and 0.1% Tween20 (blocking buffer) before incubation with antibodies. Polyclonal antibodies were used to detect mouse MCL-1 (Rockland Antibodies and Assays), BIM (Enzo Life Sciences), BCL-x (BD Biosciences), and human MCL-1 (s-19, Santa Cruz Biotechnology); monoclonal antibodies were used to detect mouse p53 (clone IMX25, Novocastra), p19/ARF (clone 5.C3.1, Rockland Antibodies and Assays), ERα (to detect the CreERT2 protein) (clone HC-20, Santa Cruz Biotechnology),

Actin (clone AC40, Sigma-Aldrich), and β-Actin (clone AC74, Sigma-Aldrich); and monoclonal antibodies were used to detect human p53 (clone FL393), HDM2 (clone N20), BCL-2 (clone C-2), BCL-X_L (clone H5) (all from Santa Cruz Biotechnology), β-Actin (clone AC15, Sigma-Aldrich), and HSP70 (mouse monoclonal antibody clone N6; detects both mouse and human protein) (a gift from Dr R Anderson, Peter MacCallum Cancer Research Institute, Melbourne, Australia). All antibodies were diluted in blocking buffer.

qRT–PCR analysis

Total RNA was extracted from paired untreated and tamoxifen-treated *Eμ-Myc* lymphoma cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The RNA was treated with DNase to remove contaminating DNA using the RNase-free DNase Qiagen kit according to the manufacturer's instructions. RNA quality and quantity were determined using the NanoDrop assay (Thermo Fisher Scientific). Aliquots of 1 μg of RNA were reverse-transcribed into cDNA using the SuperScript III first strand synthesis Supermix kit (Invitrogen) in a 20-μL reaction volume according to the manufacturer's instructions. The cDNA was diluted 10-fold in H_2O, and PCR amplifications of 1 μL of cDNA were performed with an Applied Biosystems 7900HT thermal cycler using TaqMan Universal PCR Mastermix and 0.5 μL of TaqMan primer/probes (both Applied Biosystems) in a 10-μL reaction volume. Assays specific for *Bcl-x* (Mm00437783) and (as an endogenous control for RNA quality/input) HMBS (Mm01143545) transcripts were performed. Three replicates of each reaction were performed. All qPCR data were analyzed using the $2^{-\Delta\Delta CT}$ method and expressed relative to the untreated sample of each pair.

IVIS imaging

Single-cell suspensions of lymphoma cells taken from the lymph nodes, spleens, or thymuses of sick *Eμ-Myc;CreERT2,Bcl-x*$^{fl/fl}$ and *Eμ-Myc;CreERT2;Mcl-1*$^{fl/fl}$ mice were transduced with a GFP-luc lentiviral vector as described above. Transduced cells were cultured for three passages on an OP9 cell feeder layer (Zúñiga-Pflücker et al. 1994) and then washed in PBS, and 4×10^5 to 1×10^6 live lymphoma cells were injected i.v. into C57BL/6-albino recipient mice. To visualize the tumor burden, 200 μL of 15 mg/mL D-luciferin potassium salt (Caliper Life Sciences) in PBS was administered to the mice by intraperitoneal (i.p.) injection. Mice were then anaesthetized with isoflurane inhalant and imaged using the IVIS live-imaging system (Perkin Elmer) to detect luciferase bioluminescence exactly 15 min after administration of the luciferin substrate. The tumor burden was quantified by measuring the total photon flux per second emitted from a region of interest (ROI) drawn around the whole mouse. When the lymphoma burden was sufficiently high (photon flux per second of $>1 \times 10^7$), some mice from each cohort were administered tamoxifen by oral gavage once per day for three consecutive days, and the tumor regression/progression was monitored by further bioluminescence imaging at indicated time points.

Human p53 cDNA sequence analysis

The human *p53* gene status was determined by sequencing of cDNA (Lindstrom et al. 2001). Total RNA was extracted from cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The cDNA was made from total RNA using the Advantage one-step RT–PCR kit according to the manufacturer's instructions (Clontech). *p53* was sequenced from pooled PCR reactions using the following primers: p53F1 (5′-GAAGGATCCGAGGAGCCGCAGT-3′), p53F781 (5′-CTGAGGTTGGCTCTGACTGTACCACCATCC-3′), p53R789 (5′-AACAAGCTTATTACCACTGGAGTCTTC-3′), and p53R1179 (5′-AGGGAATTCAGTCTGAGTCAGGC-3′).

DNA extraction, PCR, and DNA sequence analysis

DNA was extracted from cells using the Qiagen DNEasy tissue kit (Qiagen) according to the manufacturer's instructions, and the DNA concentration was quantified using the Nanodrop assay. Exons 4, 5–6, 7, 8–9, and 10 of the mouse *p53* gene were PCR-amplified from 100 ng of DNA using GoTaq green master mix (M712, Promega) and 3.3 μM primers using 32 cycles of 1 min at 94°C, 1 min at 52°C, and 1 min at 72°C followed by a 5-min extension at 72°C. DNA sequence analysis was performed by Micromon Monash. The following primer combinations were used to PCR-amplify and sequence mouse *p53*: Ex4F (5′-GGTTCTTCTTTGTCCCATCC-3′) with Ex4R (5′-GAGGCATTGAAAGGTCACAC-3′), Ex5F 5′-TTAGTTCCCCACCTTGACAC-3′) with Ex6R (5′-AGGCTGGAGTCAACTGTCTC-3′), Ex7F (5′-TAGTGAGGTAGGGAGCGACTTC-3′) with Ex7R (5′-CCAAGAGGAAACAGAGGAGG-3′), Ex8F (5′-CTTCTCGGGGTTCCTGTAAC-3′) with Ex9R (5′-CCTGGCAACCTGCTAATAAC-3′), and Ex10F (5′-AAACCTGTAAGTGGAGCCAG-3′) with Ex10R (5′-AGTCAGTTCTCGTAGGGTGC-3′). The sequence alignment for murine *p53* was performed using Ensembl transcript number ENSMUST00000108658 as the reference transcript.

DNA PCR to detect Mcl-1^{fl}*, Mcl-1*^{wt}*, and recombined Mcl-1 alleles*

The wild-type, *Mcl-1*^{fl}, and recombined *Mcl-1*^{fl} alleles were PCR-amplified from 100 ng of DNA using GoTaq green master mix (M712, Promega) and 0.5 μM primers using 30 cycles of 40 sec at 94°C, 30 sec at 55°C, and 1 min at 72°C followed by a 5-min extension at 72°C. The following primers were used to detect *Mcl-1*^{fl} and *Mcl-1*^{wt} alleles: 5′-GCACAATCCGTCCGCGAGCCAA-3′ and reverse 5′-GCCGCAGTACAGGTTCAAG-3′. The wild-type PCR product was smaller than the floxed PCR product. The following primers were used to detect recombined *Mcl-1*^{fl} alleles: 5′-CGACACAGATCAGCAGGCGTTC-3′ with 5′-GAGTCAGCGCGATCATTCAGCT-3′.

DNA PCR to detect homozygous deletion of the CDKN2A (INK4A/ARF) locus

The following PCR primers were used: exon 1β, (F, 5′-TGCAGTTAAGGGGGCAGGAG-3′; R, 5′-TTATCTCCTCCTCCTCCTAGCCTG-3′) and exon 2 (551R, 5′-TCTGAGCTTTGGAAGCTCT-3′; 42F, 5′-GGAAATTGGAAACTGGAAGC-3′). A GAPDH exon 8 fragment served as control for integrity of DNA.

Acknowledgments

We thank G. Siciliano and his team for help with animal husbandry, C. McLean for editorial assistance, Dr. J.M. Adams and Dr. S. Cory for advice and discussions, and Dr. Kelly Rogers for help with imaging of mice. This work is supported by a Kay Kendall Leukemia Fund Intermediate Fellowship (KKL331) and an EMBO short-term fellowship (both awarded to G.L.K.); the National Health and Medical Research Council, Australia (program grant 1016701 and fellowship 1020363 to A.S., project grant APP1049720 to M.J.H., project grant APP1041936 to W.D.F., and career development fellowship APP1024620 to E.F.L.); Cancer Research UK (program grant awarded to L.F., M. Rowe, and

A.B.R.); the Leukemia Foundation; the Leukemia and Lymphoma Society (specialized center of research 7001-13); and a German Research Council Fellowship (He 5740/1-1 awarded to M.J.H.). This work was made possible through Victorian State Government Operational Infrastructure Support and Australian Government National Health and Medical Research Council Independent Research Institutes Infrastructure Support Scheme.

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